



## Lamination of the cerebral cortex is disturbed in *Gli3* mutant mice

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### ABSTRACT

The layered organization of the cerebral cortex develops in an inside-out pattern, a process which is controlled by the secreted protein reelin. Here we report on cortical lamination in the *Gli3* hypomorphic mouse mutant *Xt<sup>l</sup>/Pdn* which lacks the cortical hem, a major source of reelin<sup>+</sup> Cajal Retzius cells in the cerebral cortex. Unlike other previously described mouse mutants with hem defects, cortical lamination is disturbed in *Xt<sup>l</sup>/Pdn* animals. Surprisingly, these layering defects occur in the presence of reelin<sup>+</sup> cells which are probably derived from an expanded *Dbx1*<sup>+</sup> progenitor pool in the mutant. However, while these reelin<sup>+</sup> neurons and also Calretinin<sup>+</sup> cells are initially evenly distributed over the cortical surface they form clusters later during development suggesting a novel role for *Gli3* in maintaining the proper arrangement of these cells in the marginal zone. Moreover, the radial glial network is disturbed in the regions of these clusters. In addition, the differentiation of subplate cells is affected which serve as a framework for developing a properly laminated cortex.

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### Introduction

The cerebral cortex as the main centre for all higher cognitive functions develops a layered structure which is essential for its function. This lamination develops in an inside-out fashion and requires the secreted glycoprotein reelin (Tissir and Goffinet, 2003). Lack of functional *reelin*, as in the *reeler* mouse mutant (Curran and D'Arcangelo, 1998; D'Arcangelo et al., 1995; Ogawa et al., 1995) and in human congenital lissencephaly patients (Hong et al., 2000) or interference with reelin signalling (Howell et al., 1997; Sheldon et al., 1997; Trommsdorff et al., 1999) result in inversion of cortical layers and in abnormally dispersed cells.

In contrast to the well understood role of reelin signalling, relatively little is known about the identity of the reelin<sup>+</sup> cell population directing cortical lamination. *Reelin* is expressed at high levels in CR cells, a major cell population in the MZ (D'Arcangelo et al., 1995; Meyer and Wahle, 1999; Ogawa et al., 1995). However, only indirect evidence supports a role for CR cells in controlling cortical layering (Ringstedt et al., 1998; Super et al., 2000). The understanding of such a role is further complicated by the existence of several reelin<sup>+</sup> cell populations with different sites of origins, migration routes, destination and molecular profiles. Some CR cells are generated from *Dbx1*<sup>+</sup> progenitor cells in the septum and in the

ventral pallium at the pallial/subpallial boundary (PSB) which express *reelin* but not *p73* and preferentially populate the rostroventral and ventral cortex (Bielle et al., 2005). Consistently, ablation of *Dbx1* progenitor cells predominantly leads to cytoarchitectural defects in the lateral cortex (Bielle et al., 2005). In addition, the caudomedial wall of the telencephalon, including the cortical hem, is a major source of a CR cell population which is characterized by the expression of reelin, *p73*, Calretinin and glutamate (Meyer et al., 2002; Takiguchi-Hayashi et al., 2004). The role of these CR cells in cortical layering has recently been tested by genetic inactivation of *p73* and by ablation of the hem (Meyer et al., 2004; Yoshida et al., 2006). Surprisingly, both mutants show normal cortical lamination except for the caudal cortex, suggesting that hem derived CR cells are not required to control cortical layering and that other sources of reelin, particularly cortical plate (CP) interneurons, are sufficient to allow lamination to proceed (Yoshida et al., 2006).

The *Gli3* mouse mutant extra-toes (*Xt<sup>l</sup>*) represents another mouse mutant with defective cortical hem development. This mutant shows severe defects in patterning the dorsal telencephalon (Fotaki et al., 2006; Grove et al., 1998; Kuschel et al., 2003; Theil et al., 1999; Tole et al., 2000a) and in preplate differentiation (Theil, 2005). In particular early cortical layering, i.e. the formation of the subplate (SP) and the marginal zone (MZ) is severely disrupted in these animals making it difficult to study cortical lamination. To circumvent these difficulties, we made use of the compound *Gli3* hypomorphic mouse mutant *Xt<sup>l</sup>/Pdn* which shows much milder regionalization defects. This mutant lacks the cortical hem and consequently contains few cortical reelin<sup>+</sup>/*p73*<sup>+</sup> CR cells but has

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numerous *reelin*<sup>+</sup>/*p73*<sup>-</sup> CR cells probably arising from an expanded *Dbx1*<sup>+</sup> progenitor pool. Despite the presence of these *reelin*<sup>+</sup> cells, however, cortical lamination is disturbed in the mutant. These *reelin*<sup>+</sup> cells form dense cell clusters and the radial glial scaffold is severely disturbed at the sites of these clusters. Our analysis therefore suggests a role for *Gli3* in regulating cortical lamination by maintaining an even distribution of CR neurons over the cortical surface.

## Materials and methods

### Mice

*Xt*<sup>l</sup> and *Pdn* heterozygous animals were kept on a mixed C57Bl6/C3H and C3H/He background, respectively, and were interbred. Embryonic (E) day 0.5 was assumed to start at midday of the day of vaginal plug discovery. *Xt*<sup>l</sup>/*Pdn* embryos were readily distinguished from heterozygous and wild-type embryos by forebrain and/or limb morphology (Kuschel et al., 2003). For each marker and each stage, 3–5 different, non-

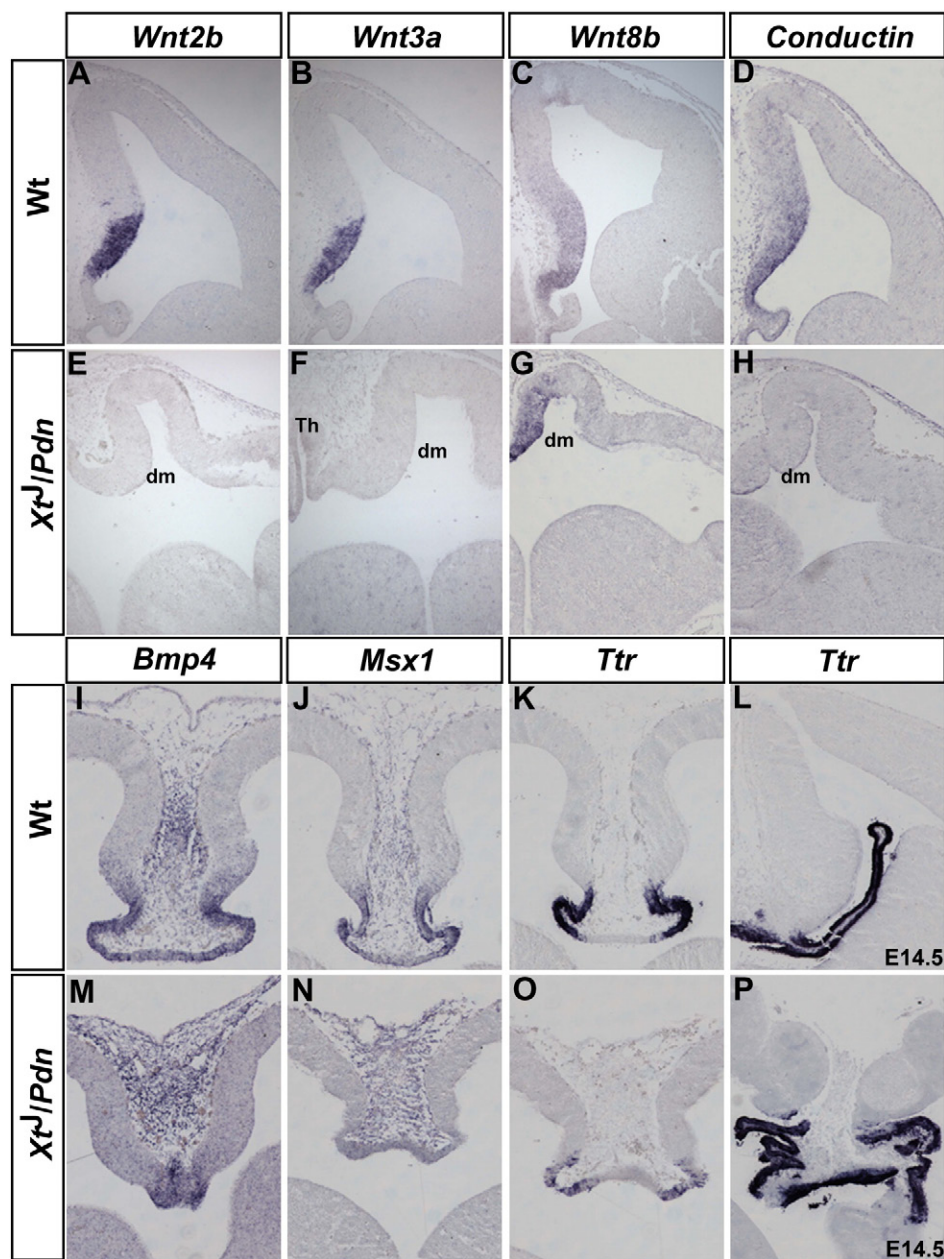
exencephalic embryos were analysed at rostral, medial and caudal levels of the developing cortex.

### Explant culture of telencephalic tissue

The dorsal telencephalon or the neocortex of E10.5 wildtype and *Gli3* mutant embryos was dissected in HBSS and the surface ectoderm removed manually. Explants were cultured on Millicell-CM culture plate inserts (Millipore, #PICMORG50) in organ culture dishes. Culture medium was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal calf serum, 1× non-essential amino acids (GIBCO), 1 mM sodium pyruvate (GIBCO) and 1× streptomycin/penicillin (GIBCO). Tissue pieces were maintained under in vitro conditions for 48 h and then processed for in situ hybridization.

### In situ hybridization and immunohistochemistry

Antisense RNA probes for *Bmp4* (Jones et al., 1991), *Conductin* (Lustig et al., 2002), *Cux2* (Zimmer et al., 2004), *Dbx1* (Yun et al., 2001), *Dlx2* (Bulfone et al., 1993), *ER81*



**Fig. 1.** Dorsal midline defects in *Xt*<sup>l</sup>/*Pdn* embryos. In situ hybridization analysis on coronal sections through the brains of wild-type (A–D, I–L), and *Xt*<sup>l</sup>/*Pdn* (E–H, M–P) embryos. (A–C, E–G) Cortical hem expression of *Wnt2b*, *Wnt3a* is absent in *Xt*<sup>l</sup>/*Pdn* embryos while *Wnt8b* expression remains in the highly abnormal dorsomedial region. (D, H) *Xt*<sup>l</sup>/*Pdn* mutants lack *Conductin* expression in the dorsomedial telencephalon. (I, J, M, N) *Bmp4* expression and signalling as indicated by *Msx1* expression is reduced in the dorsal telencephalon. (K, L, O, P) Delay of choroid plexus development in *Xt*<sup>l</sup>/*Pdn* embryos. While only few cells express *Ttr* at reduced levels at E12.5, *Ttr* expression has recovered at E14.5. Note the disorganized structure of the choroid plexus. dm: dorsomedial telencephalon, Th: thalamus.



(Lin et al., 1998), *Msx1* (Hill et al., 1989), *Ngn2* (Gradwohl et al., 1996), *p73* (XM 131858; GenBank), *reelin* (D'Arcangelo et al., 1995), *RORβ* (Hevner et al., 2003a), *Sfrp2* (Kim et al., 2001), *Shh* (Echelard et al., 1993), *Sox5* (Lefebvre et al., 1998), *Ttr* (Duan et al., 1989), *Wnt2b* (Grove et al., 1998), *Wnt3a* (Roelink and Nusse, 1991) and *Wnt8b* (Richardson et al., 1999) were DIG labelled. In situ hybridization on tissue explants and on 12 µm serial paraffin sections of mouse embryos were performed as described (Kuschel et al., 2003; Theil, 2005).

Immunohistochemical analysis was performed as described previously (Theil, 2005) using the following antibodies: BrdU (1:20; Bio-Science), Calbindin (1:2000; Swant), Calretinin (1:2000; Chemicon), Calretinin (1:1000; Swant), CS56 (1:1000; Sigma), Foxg1 (1:50; Abcam), MAP2 (1:1000; Sigma), Nestin (1:100; DSHB), RC2 (DSHB; 1:50), reelin (G10; 1:200), Tbr1 (1:2500) (Englund et al., 2005).

For birth dating analysis of cortical lamination, pregnant females received a single, intraperitoneal injection of BrdU (10 mg/ml) at E11.5, E13.5 or E15.5 and embryos were collected at P0. For BrdU-immunocytochemistry, slides were incubated in 0.1 M Na<sub>4</sub>B<sub>4</sub>O<sub>7</sub> after denaturing with 2 N HCl. For immunohistochemistry/RNA in situ hybridization, sections were washed after RNA detection for 24 h in PBS and then processed for immunohistochemistry as described above.

## Results

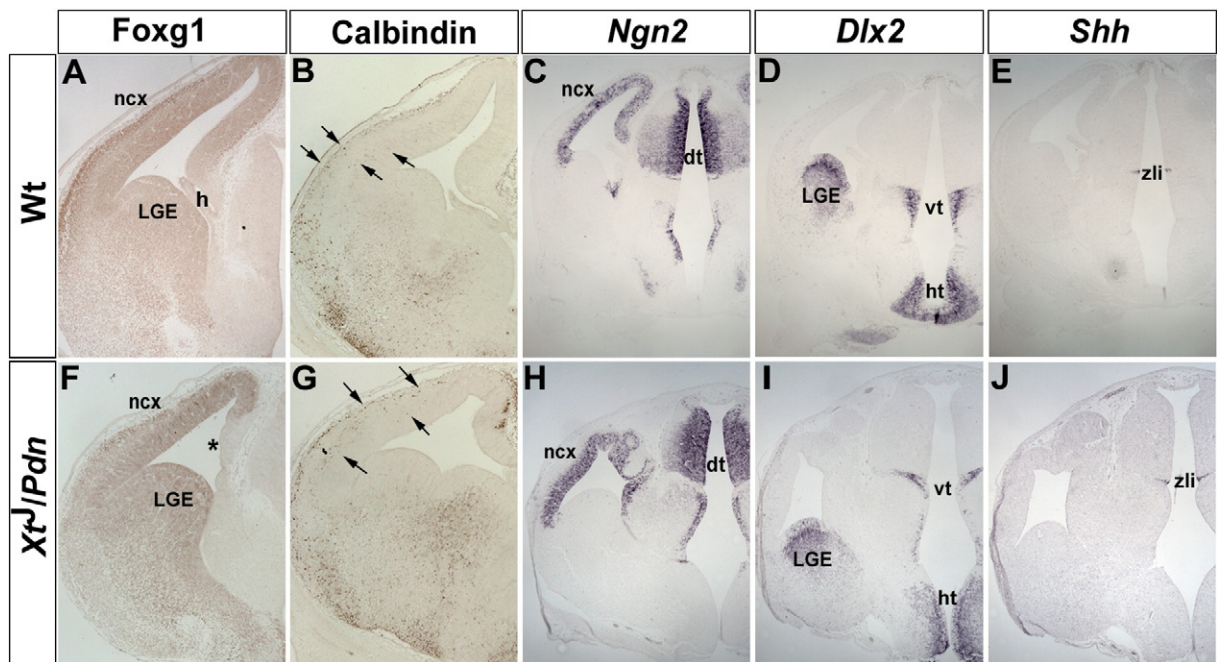
### Regionalization defects in the telencephalon of *Xt<sup>l</sup>/Pdn* embryos

Previously, we reported that the early cortical layering, i.e. the formation of the preplate and its derivatives, the MZ and the SP, is severely affected in *extra-toes* (*Xt<sup>l</sup>*) embryos (Theil, 2005) in which a deletion removes all *Gli3* sequences 3' of the second zinc finger (Büscher et al., 1998). Due to the severity of the phenotype, however, it is difficult to analyze the proper lamination process in these animals. We therefore focussed on the *Gli3* hypomorphic mouse *Xt<sup>l</sup>/Pdn* (Kuschel et al., 2003; Schimmang et al., 1994) in which the *Gli3* transcript levels are reduced due to the integration of a retrotransposon (Thien and Rüther, 1999). Before analyzing cortical lamination we started to define the extent to which early patterning of the telencephalon is affected in this mutant. Our previous whole mount in situ hybridization analysis of E12.5 forebrain development in these mutants had indicated the absence of *Wnt* gene expression in the dorsal telencephalic midline of these animals (Kuschel et al., 2003) which we could also confirm at E10.5

(data not shown). To analyze dorsal midline and in particular hem development more accurately, we performed a *Wnt* expression analysis on sectioned telencephalic tissue. While the expression of *Wnt3a* and *Wnt2b* was readily detectable in the wild-type cortical hem, the expression of these hem markers was absent from the *Xt<sup>l</sup>/Pdn* mutant cortex (Figs. 1A, B, E, F). In contrast, *Wnt8b* expression which occurs in the hem and in the hippocampal anlage of wild-type embryos was still detected in dorsomedial structures of the E12.5 *Xt<sup>l</sup>/Pdn* cortex (Figs. 1C, G). This residual *Wnt8b* expression, however, did not activate canonical *Wnt* signalling as indicated by the absence of *Conductin* expression (Figs. 1D, H).

In addition to several *Wnt* genes, the dorsal midline is also positive for several *Bmp* genes which are essential for development of the choroid plexus (Hebert et al., 2002). Interestingly, we detected weak expression of *Bmp4* in the dorsomedial telencephalon of *Xt<sup>l</sup>/Pdn* embryos (Figs. 1I, M). We also observed weak expression of *Msx1* suggesting that *Bmp* signalling is weakly activated in the mutant (Figs. 1J, N). Consistent with this, *Ttr* a choroid plexus marker (Duan et al., 1989) showed a patchy and considerably weaker expression in the dorsal midline region of the E12.5 *Xt<sup>l</sup>/Pdn* mutant (Figs. 1K, O). At E14.5, however, *Ttr* was strongly expressed in a highly dysmorphic choroid plexus (Figs. 1L, P). This analysis therefore suggests the absence of the cortical hem but residual *Bmp* signalling from the *Xt<sup>l</sup>/Pdn* telencephalic roof coinciding with an abnormal development of the choroid plexus.

In addition to dorsal midline defects, the formation of the boundary between dorsal telencephalon and diencephalon is affected in *Xt<sup>l</sup>/Xt<sup>l</sup>* mutant (Theil et al., 1999) resulting in the juxtaposition of the neocortex and the eminentia thalamica and to a mixing of cells derived from both tissues (Fotaki et al., 2006). We therefore analyzed whether *Xt<sup>l</sup>/Pdn* embryos show a similar defect. A morphological inspection of the telencephalon in E12.5, E14.5 and E18.5 embryos indicated a dysmorphic dorsomedial telencephalon but suggested a separation of dorsal telencephalon and diencephalon. To further analyze this we used various markers characteristic for specific subdivisions of the forebrain. In wildtype embryos, Foxg1 is expressed



**Fig. 2.** Dorsal telencephalon and diencephalon are morphologically separated in *Xt<sup>l</sup>/Pdn* embryos. Immunohistochemical and in situ hybridization analysis on E12.5 wildtype (A–E) and *Xt<sup>l</sup>/Pdn* (F–J) using the indicated markers. (A, F) Foxg1 is expressed in the telencephalon except for the cortical hem (h) in wildtype and the highly abnormal dorsomedial region of the *Xt<sup>l</sup>/Pdn* mutant embryo. (B, G) Calbindin stains interneurons originating in the LGE and migrating into the neocortex in both genotypes. *Ngn2* (C, H) and *Dlx2* (D, I) staining reveals the dorso/ventral subdivisions of the telencephalon and diencephalon. (E, J) *Shh* expression marks the zona limitans intrathalamica (zli).

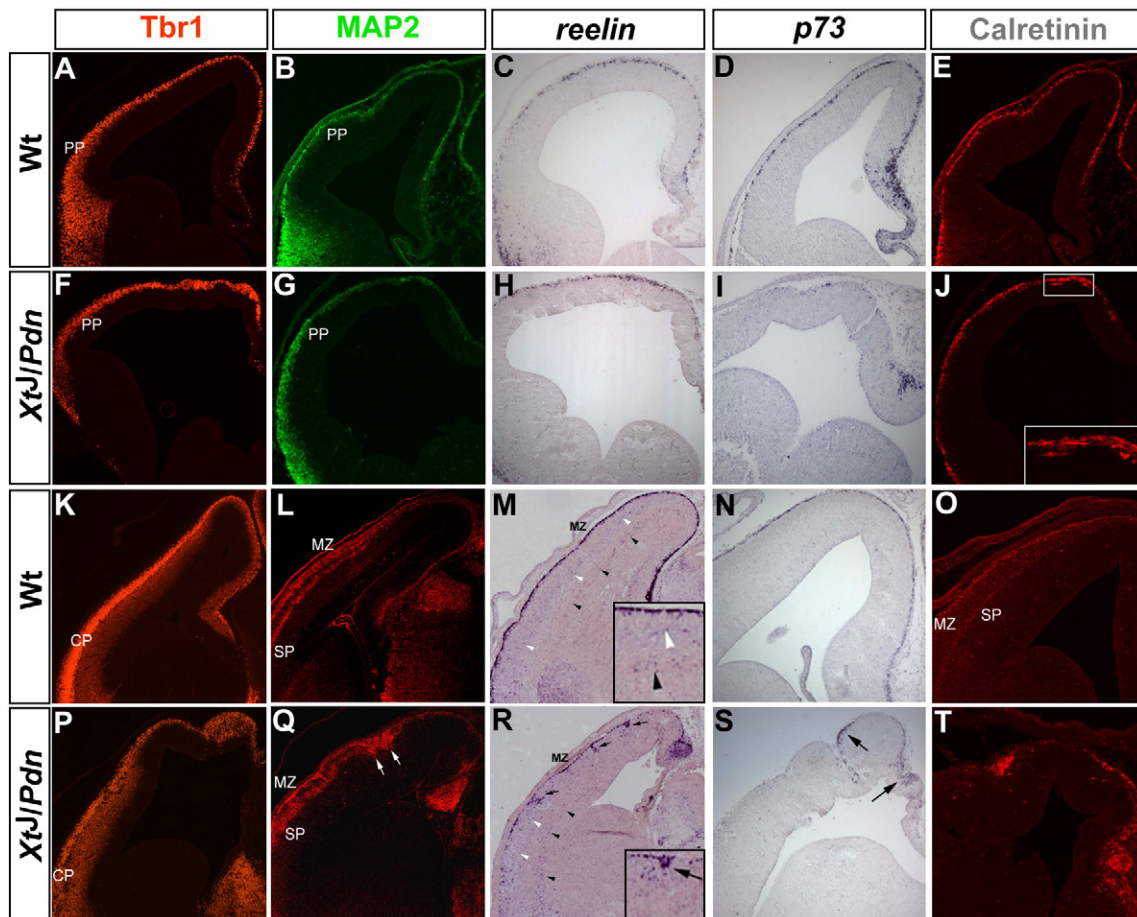
by all telencephalic cells except for the cortical hem and for CR neurons (Hanashima et al., 2002; Tao and Lai, 1992). This expression pattern is maintained in the mutant where only the dorsomedial region is negative for Foxg1 (Figs. 2A, F). In contrast to *Xt<sup>l</sup>/Xt<sup>l</sup>* embryos, we did not notice the presence of Foxg1<sup>−</sup> cells within the *Xt<sup>l</sup>/Pdn* neocortex. Similarly, Calbindin immunostaining did not reveal cell clusters in the neocortex (Figs. 2B, G) as described for *Xt<sup>l</sup>/Xt<sup>l</sup>* embryos (Fotaki et al., 2006). Finally, in situ hybridization for *Ngn2*, *Dlx2* and *Shh* indicated the presence of the major subdivisions of the *Xt<sup>l</sup>/Pdn* telencephalon and diencephalon (Figs. 2C–E, H–J). These data suggest that the development of the dorsomedial telencephalon is severely affected in both, *Xt<sup>l</sup>/Xt<sup>l</sup>* and *Xt<sup>l</sup>/Pdn* embryos, but that the latter show a morphological and molecular separation of telencephalon and diencephalon.

#### The *Xt<sup>l</sup>/Pdn* cortex shows a severe reduction in hem derived CR cells

Our previous analysis showed that preplate development and differentiation are severely affected in *Xt<sup>l</sup>/Xt<sup>l</sup>* embryos (Theil, 2005). We therefore analyzed this process in *Xt<sup>l</sup>/Pdn* embryos. The transcription factor *Tbr1* is expressed in the preplate and is essential for its differentiation (Hevner et al., 2001). Immunohistochemical analysis revealed a continuous band of *Tbr1*<sup>+</sup> cells at the outer part of the developing cortex in E12.5 and E14.5 wild-type and in *Xt<sup>l</sup>/Pdn* embryos

although the mutant preplate appeared undulated dorsomedially (Figs. 3A, F, K, P). Similarly, the expression of MAP2 which labels preplate neurons and the SP/MZ at E12.5 and E14.5, respectively, was unaffected in the E12.5 *Xt<sup>l</sup>/Pdn* cortex but the mutant SP and MZ appeared more diffuse at E14.5. In addition, several MAP2<sup>+</sup> cell clusters were detected in the medial neocortex (Figs. 3B, G, L, Q).

We next used in situ hybridization analysis to gain insights into the cellular composition of the preplate and its derivatives. Hem derived CR cells express *reelin*, *p73* and Calretinin (Alcantara et al., 1998; del Rio et al., 1995; Hevner et al., 2003b; Meyer et al., 2004, 1999, 2002; Ogawa et al., 1995; Soda et al., 2003). This analysis showed a single layer of *reelin* expressing cells at the entire wild-type E12.5 and E14.5 cortical surface (Figs. 3C, M). While in E12.5 *Xt<sup>l</sup>/Pdn* embryos the layer of *reelin* expressing cells appeared relatively normal (Fig. 3H), *reelin* expressing cells formed small aggregates in the dorsomedial E14.5 mutant neocortex (Fig. 3R) which are reminiscent of the large clusters of *reelin* expressing cells in the *Xt<sup>l</sup>/Xt<sup>l</sup>* cortex (Theil, 2005). In addition, *reelin* expression was detected at low levels in two rows of cells within the wild-type E14.5 neocortex which correspond to migrating interneurons and which have recently been suggested to play an important role in cortical lamination (Alcantara et al., 2006; Yoshida et al., 2006). Distinct rows of *reelin* expressing neurons were only detected in the mutant lateral neocortex while more medial regions showed an



**Fig. 3.** Development of the preplate and its differentiation in *Xt<sup>l</sup>/Pdn* embryos. Immunohistochemical and in situ hybridization analysis of wild-type (A–E, K–O) and *Xt<sup>l</sup>/Pdn* embryos (F–J, P–T) on coronal sections through E12.5 (A–J) and E14.5 (K–T) forebrains. (A, F) High *Tbr1* expression levels mark preplate neurons and the cortical plate in wild-type embryos. (F, P) While the *Tbr1* expression domain at E12.5 appears thinner dorsomedially, E14.5 *Xt<sup>l</sup>/Pdn* embryos lack *Tbr1* expression in dorsomedial regions. (B, L) Anti-MAP2 immunostaining characterizing preplate neurons (B) and the MZ and SP at E14.5 (L). MAP2 expression is unaffected in E12.5 *Xt<sup>l</sup>/Pdn* embryos but appears disorganized at E14.5. (C, D, M, N) *reelin* and *p73* expression in a single row of cells in the wild-type telencephalon. (H, R) *reelin* expressing cells are less densely packed and form small clusters (arrows) in the E14.5 mutant cortex. (I, S) Nearly complete lack of *p73* expression in the neocortex of *Xt<sup>l</sup>/Pdn* embryos. (E) Calretinin<sup>+</sup> cells form a single, continuous row of cells on the E12.5 basal cortical surface. (J) Calretinin<sup>+</sup> cells in the mutant cortex. The inset shows the formation of a multilayered structure. (O) Calretinin labels the MZ and SP. (T) Calretinin<sup>+</sup> cells cluster in the mutant.



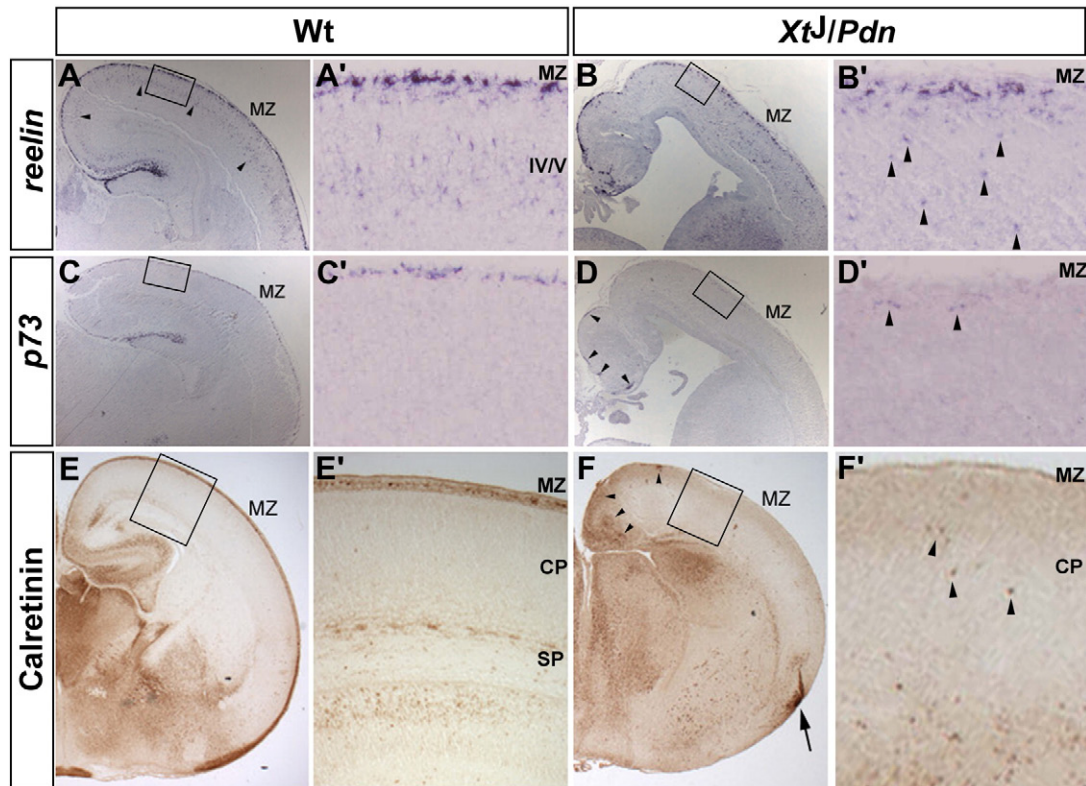
uneven distribution of *reelin* in the CP. In contrast to the less severely affected *reelin* expression, *p73* expression was drastically altered in *Xt<sup>l</sup>/Pdn* embryos. Like *reelin*<sup>+</sup> cells, *p73* expressing cells covered the cortical surface of wild-type embryos (Figs. 3D, N) whereas E12.5 *Xt<sup>l</sup>/Pdn* embryos had only a few *p73* expressing cells in the neocortical MZ and a cluster of cells in the dorsomedial telencephalon. A similar distribution of *p73* expressing cells was observed in E14.5 *Xt<sup>l</sup>/Pdn* embryos (Fig. 3S). Taken together with the altered *reelin* expression this finding suggests a severe reduction in the hem derived *reelin*<sup>+</sup>/*p73*<sup>+</sup> CR subpopulation and a concomitant increase in a *reelin*<sup>+</sup>/*p73*<sup>−</sup> CR cell population similar to our previous observations in *Xt<sup>l</sup>/Xt<sup>l</sup>* embryos (Theil, 2005). Finally, immunohistochemical analysis of Calretinin expression, a marker for CR and pioneer neurons, showed few abnormalities in E12.5 *Xt<sup>l</sup>/Pdn* embryos. As in wild-type embryos, Calretinin<sup>+</sup> neurons formed a single layer except for the dorsomedial telencephalon where several layers were detected (Figs. 3E, J). In contrast, at E14.5, Calretinin<sup>+</sup> neurons formed dense clusters dorsomedially but were absent in the lateral MZ similar to *p73* expression (Figs. 3O, T). This analysis suggests that the formation of the preplate is not affected in *Xt<sup>l</sup>/Pdn* embryos, but that the MZ shows a marked reduction of hem derived *reelin*<sup>+</sup>/*p73*<sup>+</sup>/Calretinin<sup>+</sup> CR neurons and a clustering of *reelin*<sup>+</sup> and Calretinin<sup>+</sup> cells.

Interestingly, similar alterations in the cellular composition of the MZ were observed in newborn mutants. In wild-type newborn animals, strong *reelin* expression was detected in Cajal Retzius cells within the MZ (Fig. 4A). An additional weak expression was detected in the lower cortical plate at the level of layer IV/V (Yoshida et al., 2006). In *Xt<sup>l</sup>/Pdn* animals we could identify fewer *reelin* expressing cells in the MZ (Fig. 4B). Also, a band of *reelin* expressing cells was observed in the lateral CP but not in the medial neocortex where these

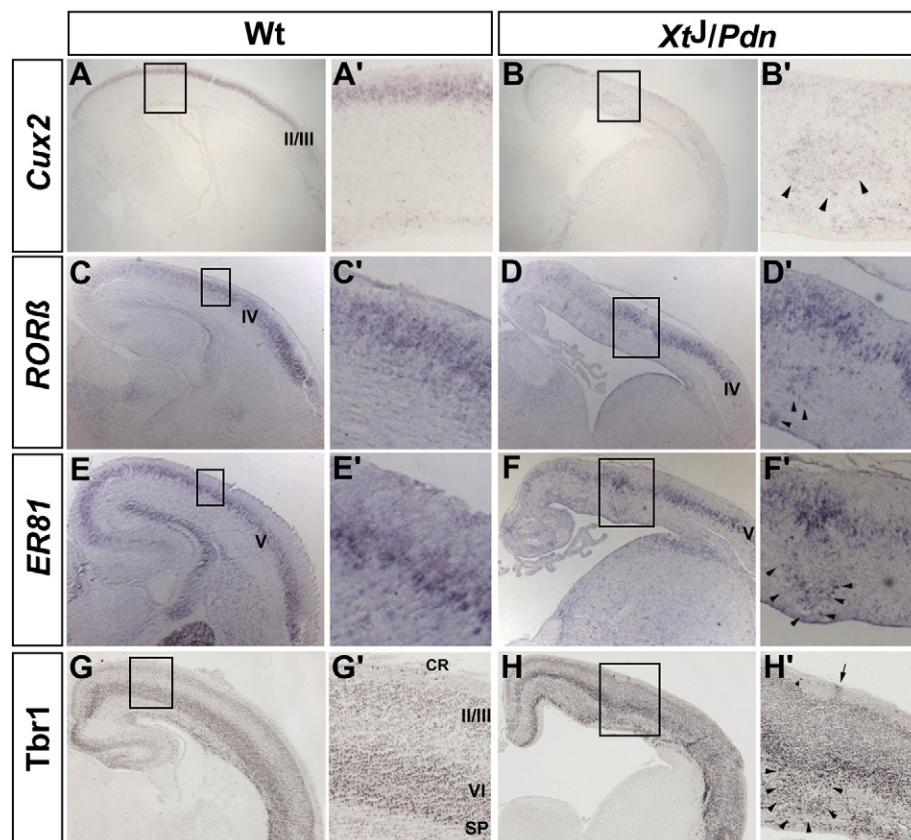
cells showed a strong dispersal (Fig. 4B). In contrast to the numerous *reelin* expressing cells in the MZ, few *p73*<sup>+</sup> cells were found in the *Xt<sup>l</sup>/Pdn* neocortical MZ while groups of *p73* expressing cells were found in the highly dysmorphic dorsomedial cortex (Figs. 4C, D). Finally, Calretinin<sup>+</sup> cells were detected in the wildtype MZ and SP while Calretinin expression was absent from the mutant MZ and SP (Figs. 4E, F). The ectopic Calretinin expression in the lateral cortical MZ corresponds to an ectopic nerve bundle (T.T., unpublished data). Taken together these data indicate that hem derived CR cells (*p73*<sup>+</sup>; *reelin*<sup>+</sup>; Calretinin<sup>+</sup>) are nearly absent while *reelin*<sup>+</sup>/*p73*<sup>−</sup> cells are found in the medial and lateral cortex.

#### Neocortical lamination in *Xt<sup>l</sup>/Pdn* embryos

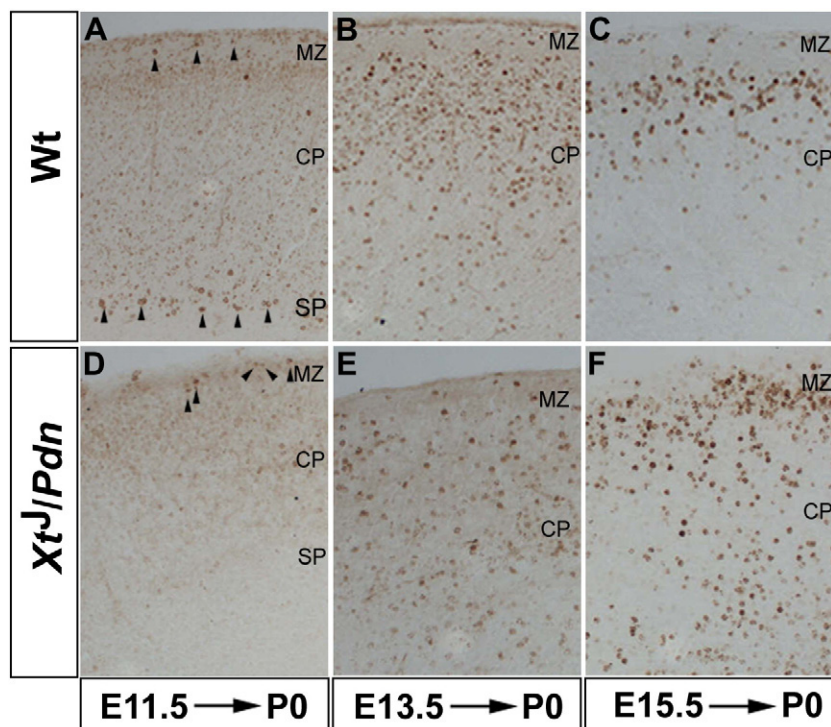
Given these alterations in the MZ and its importance for the cortical lamination process (Super et al., 1998) we used immunohistochemistry and in situ hybridization analysis to characterize the developing neocortical layers in *Xt<sup>l</sup>/Pdn* animals. As these pups die shortly after birth this analysis was confined to the P0 stage. The transcription factors *Cux2*, *RORβ*, and *ER81* distinguish emerging layers II/III, IV and V, respectively (Figs. 5A, C, E). In the *Xt<sup>l</sup>/Pdn* mutants, we could identify regions in the medial (*Cux2*) or lateral neocortex (*RORβ*, and *ER81*) which show a layered expression of these markers, though cells expressing these genes are more dispersed within these domains. In addition, we also identified regions where the cortical layering is severely disturbed. In these areas, the *Cux2*, *RORβ* and *ER81* expression domains are undulated and discontinuous. Cells expressing these genes were even found close to the ventricular surface (Figs. 5B, D, F). Finally, the *Tbr1* transcription factor shows high level expression in the SP, layer VI and CR neurons and weaker expression levels in layer II/III neurons



**Fig. 4.** Development of the MZ and subplate is affected in *Xt<sup>l</sup>/Pdn* embryos. Coronal sections through the brains of newborn wild-type (A, C, E) and *Xt<sup>l</sup>/Pdn* mutant animals (B, D, F). (A', B' C', D', E and F') Higher magnifications of the boxed areas in A, B, C, D, E and F. (A, B) Fewer *reelin* expressing cells in the MZ are found in the *Xt<sup>l</sup>/Pdn* brains. *Reelin*<sup>+</sup> cells are not so densely packed as in wild-type embryos. Also note the more dispersed distribution of weakly *reelin* expressing cells in layer IV/V (arrowheads). (C, D) *p73* expression is strongly reduced in the mutant MZ except for the dorsomedial most area (arrowheads). (E, F) The *Xt<sup>l</sup>/Pdn* neocortex lacks Calretinin staining in the MZ. The arrow in F marks an ectopic cluster of Calretinin positive fibers.



**Fig. 5.** Cortical lamination defects in *Xtl/Pdn* embryos. In situ hybridization and immunofluorescence analysis on coronal sections of newborn wild-type (A, C, E, G) and *Xtl/Pdn* animals (B, D, F, H). (A, C, E) *Cux2*, *RORβ*, and *ER81* expression mark cortical laminae II/III, IV, and V of wild-type embryos, respectively. (B, D, F) In *Xtl/Pdn* embryos, the expression of these markers is more diffuse and even occurs close to the ventricular surface (see insets B', D' and F'). (G, H) *Tbr1* is strongly expressed in layer VI, SP and CR neurons and at weaker levels in layer II/III of wildtype brains. In the mutant, *Tbr1*<sup>+</sup> cells are positioned close to the ventricular surface (arrowheads). Ectopic *Tbr1* staining was also found in the MZ (arrow).



**Fig. 6.** BrdU birthdating analysis of cortical neurons. (A–F) Coronal sections at P0 stained with an anti-BrdU antibody after BrdU administration at E11.5 (A, D), E13.5 (B, E) and E15.5 (C, F). (A, D) In wild-type embryos, administration of BrdU at E11.5 labels MZ and SP neurons (arrowheads), whereas only few or no neurons are BrdU labelled in the MZ (arrowheads) and SP of *Xtl/Pdn* animals, respectively. (B, C; E, F) BrdU<sup>+</sup> neurons are found in the lower and upper cortex of wild-type animals after labelling at E13.5 (B) and E15.5 (C), respectively. (E, F) In *Xtl/Pdn* newborns, BrdU labelled neurons occupy all cortical layers.



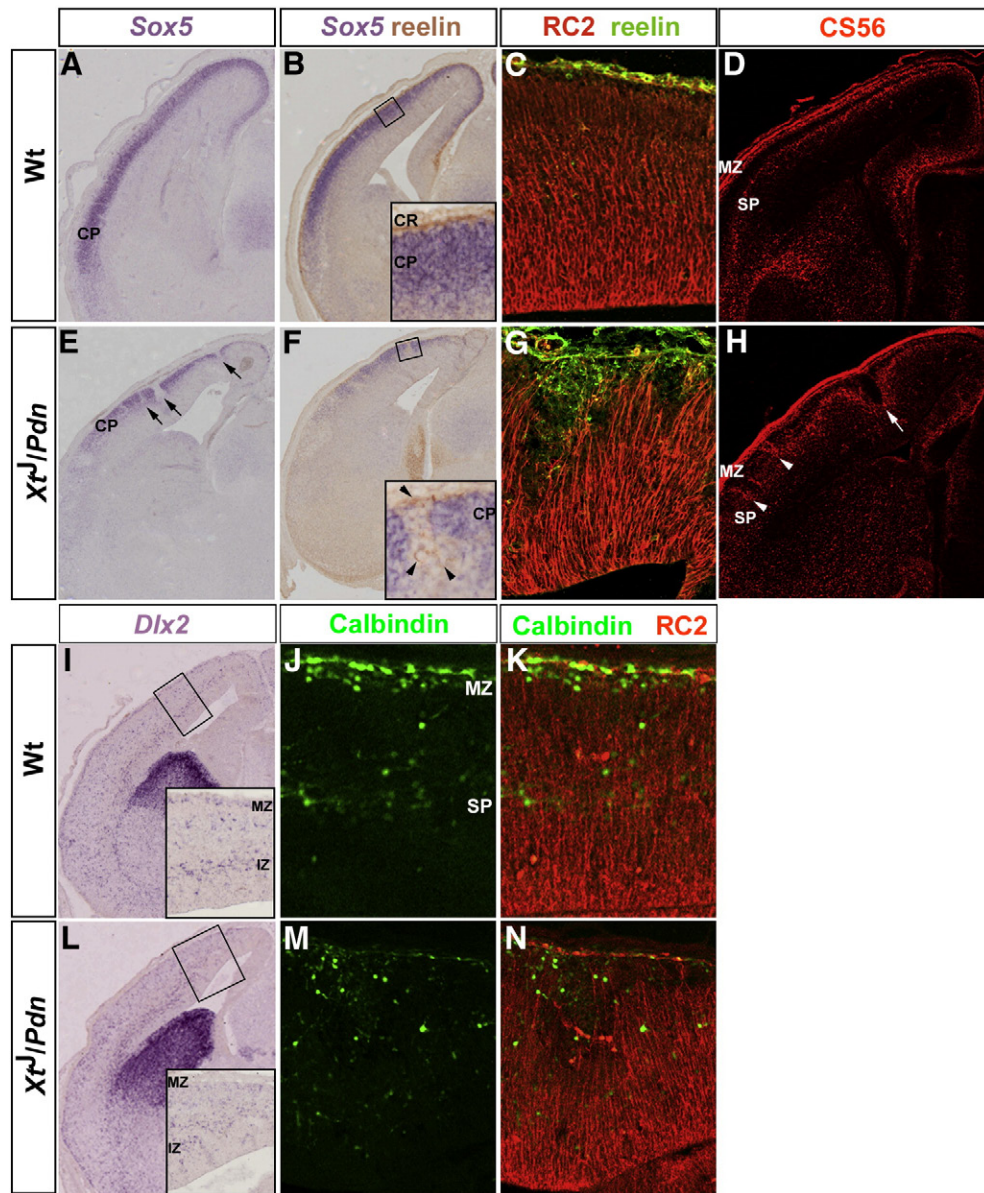
(Fig. 5G). Similar to the other markers, *Tbr1*<sup>+</sup> cells were also found in an abnormal position close to the ventricular surface (Fig. 5H). In addition, we did not observe *Tbr1* staining in the MZ characteristic of CR neurons. These results suggest that the layered neocortical organization is disturbed in the mutant.

To further analyze cortical lamination we performed a birthdating analysis of cortical neurons. To this end, we injected pregnant mice with BrdU at E11.5, E13.5 and E15.5 and examined the distribution of labeled neurons at P0 to determine the migration of cortical neurons. After labeling preplate neurons at E11.5, we detected BrdU<sup>+</sup> cells in the MZ and the SP of wild-type pups (Fig. 6A). In contrast, only few neurons were labeled in the MZ of *Xt<sup>l</sup>/Pdn* newborns consistent with the lack of Calretinin<sup>+</sup> neurons and the reduced numbers of *p73* and *reelin* expressing cells. Also, no labeling was observed in the SP (Fig. 6D). Furthermore, wild-type neurons labeled by injection at E13.5

and E15.5 predominately migrated to layers IV/V and II/III of the P0 cortex, respectively (Figs. 6B, C). Similar to our findings on lamina specific gene expression, however, BrdU<sup>+</sup> neurons showed a more dispersed distribution and in some regions did not migrate to their prospective layers but settled throughout the entire CP in the newborn *Xt<sup>l</sup>/Pdn* neocortex (Figs. 6E, F). Thus, the BrdU birthdating analysis further confirms the lamination defects in the *Gli3* mutant cortex.

#### Clusters of *reelin*<sup>+</sup> neurons disrupt CP organization

Next, we started to analyze causes for the cortical lamination defects in *Xt<sup>l</sup>/Pdn* embryos. As *reelin* is required for neocortical organization (D'Arcangelo et al., 1995; Ogawa et al., 1995; Rice and Curran, 2001), the altered distribution of *reelin*<sup>+</sup> cells and in



**Fig. 7.** Ectopic *reelin*<sup>+</sup> clusters disrupt the organization of the CP. Coronal sections through the brains of E14.5 wild-type (A–D, I–K) and *Xt<sup>l</sup>/Pdn* (E–H, L–N) embryos. (A, E) The *Sox5* expression domain exhibits gaps in the mutant CP (arrows). Also note the reduced expression levels in the medial neocortex and the complete absence of *Sox5* expression in the lateral neocortex. (B, F) In situ hybridization (*Sox5*) combined with immunohistochemical analysis (*reelin*) shows that clusters of *reelin*<sup>+</sup> neurons are located within the *Sox5* negative areas. (C, G) Immunostaining for RC2 reveals a shortening of radial glial fibers at the sites of *reelin*<sup>+</sup> clusters. Between the two *reelin*<sup>+</sup> clusters, radial glial cells extend their processes to the pial surface. (D, H) CS56 staining reveals the MZ and SP. The arrow in (H) marks the SP underneath a cluster, arrowheads CS56<sup>+</sup> cells in the CP. (I, J, K) *Dlx2* and Calbindin expression mark migrating interneurons in the wildtype neocortex. (L, M, N) The interneuron migratory routes appear more diffuse in *Xt<sup>l</sup>/Pdn* embryos. Note the absence of *Dlx2* or Calretinin clusters.

particular the clustering of these cells in the *Xt<sup>l</sup>/Pdn* mutant may interfere with proper cortical layering. To examine this possibility we first analyzed the generation of the CP in the developing mutant cortex. *Sox5* marks CP neurons in the E14.5 medial and lateral neocortex of wild-type embryos (Fig. 7A). While *Sox5* expression is nearly completely abolished in the lateral neocortex of *Xt<sup>l</sup>/Pdn* embryos the *Sox5* expression domain shows several gaps in more medial cortical areas (Fig. 7E) which correspond to the ectopic *reelin* clusters in the mutant CP (Fig. 7F). Moreover, even single ectopic *reelin*<sup>+</sup> cells in the CP are surrounded by a ring of *Sox5* expressing cells (data not shown). This complementary expression patterns suggests an exclusion of *Sox5* expressing CP neurons from the *reelin*<sup>+</sup> territories.

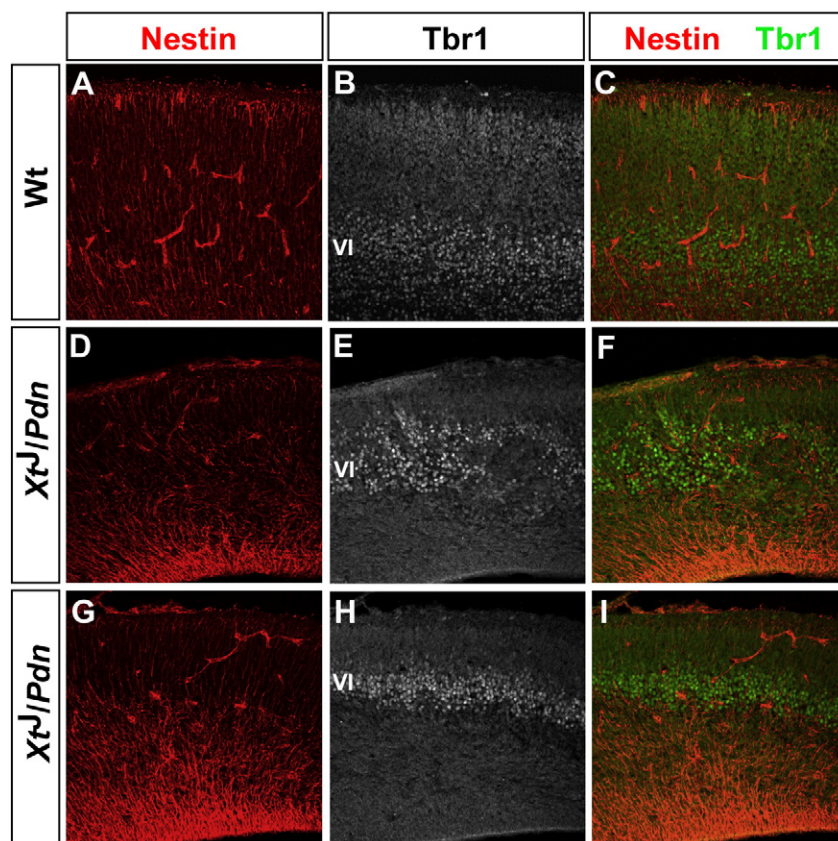
To further analyze whether the *reelin*<sup>+</sup> cell aggregates might interfere with the cortical lamination process we examined the formation of the radial glial scaffold which is essential for guiding migrating cortical neurons (Rakic, 2003). In wild-type embryos and in most parts of the mutant cortex, radial glial cells extend fibers from the ventricular to the pial cortical surface (Figs. 7C, G). However, in regions immediately underlying the *reelin*<sup>+</sup> cell clusters, the radial glial scaffold appears to be disrupted in the mutant (Fig. 7G). In these areas, radial glial fibers are severely shortened and do not reach the pial surface but end within the CP suggesting that the exclusion of *Sox5* expressing CP neurons from the *reelin* clusters is caused by this shortening of the radial glial fibers.

As the *reelin*<sup>+</sup> aggregates appear to be smaller than the actual gaps in the CP (Fig. 7F) we investigated the possibility that SP cells might be part of these clusters. The SP as well as the MZ is labelled by MAP2 and CS56. Interestingly, MAP2<sup>+</sup> cells form clusters in the E14.5 *Xt<sup>l</sup>/Pdn* neocortex (Fig. 3Q). In contrast, immunofluorescence analysis with

CS56 revealed two separate rows of cells corresponding to the MZ and the SP (Figs. 7D, H). Interestingly, the SP cells surround the lower end of a bulge located in the medial neocortex. However, the CS56 staining appeared more diffuse and we occasionally observed groups of CS56<sup>+</sup> cells in the CP.

A clustering of *reelin*<sup>+</sup> cells has been observed in transgenic mice overexpressing BDNF under the control of the nestin enhancer (Ringstedt et al., 1998). These *reelin*<sup>+</sup> aggregates form as a consequence of a segregation from clusters of GABAergic interneurons (Alcantara et al., 2006). This analysis prompted us to investigate the distribution of interneurons which are derived from the ventral telencephalon, enter the cortex by tangential migration and are marked by *Dlx2*, *Gad67* and Calbindin expression (Anderson et al., 2001; Ang et al., 2003; Nery et al., 2002). In situ hybridization for *Dlx2* and *Gad67* revealed migrating interneurons on their migratory routes in the MZ and in the intermediate zone (IZ) of the E14.5 wild-type neocortex (Fig. 7I and data not shown). In the *Xt<sup>l</sup>/Pdn* neocortex, these interneurons are more diffusely distributed, but do not cluster in the MZ (Fig. 7L). Immunofluorescence analysis for Calbindin showed a similar pattern (Figs. 7J, K, M, N) suggesting that the *reelin*<sup>+</sup> aggregates form independently of potential defects in interneuron development.

As the radial glia scaffold is disrupted at the sites of *reelin*<sup>+</sup> clusters we finally investigated whether this disruption might cause the lamination defects in *Xt<sup>l</sup>/Pdn* embryos. To this end, we performed double immunofluorescence staining for Nestin which marks the radial glia scaffold and for Tbr1 to reveal lamina organization in newborn animals. In wild-type P0 pups, the layer specific distribution of Tbr1<sup>+</sup> neurons coincides with radial glial extensions from the ventricular to the pial surface (Figs. 8A, C). In the *Gli3* mutant P0



**Fig. 8.** A disorganized radial glial network correlates with lamination defects in the *Gli3* mutant neocortex. Immunofluorescence analysis on coronal sections through the brains of wildtype (A–C) and *Xt<sup>l</sup>/Pdn* (D–I) P0 animals with the indicated antibodies. (A–C) Radial glia scaffold and distribution of Tbr1<sup>+</sup> neurons in layer VI (high level expression) and in layer II/III (low level expression). (D–F) The layer specific distribution of Tbr1<sup>+</sup> layer VI neurons is severely disturbed in regions with a disorganized radial glial network. (G–I) In regions where radial glia extensions reach the pial surface positioning of Tbr1<sup>+</sup> neurons is slightly more diffuse than in wild-type.



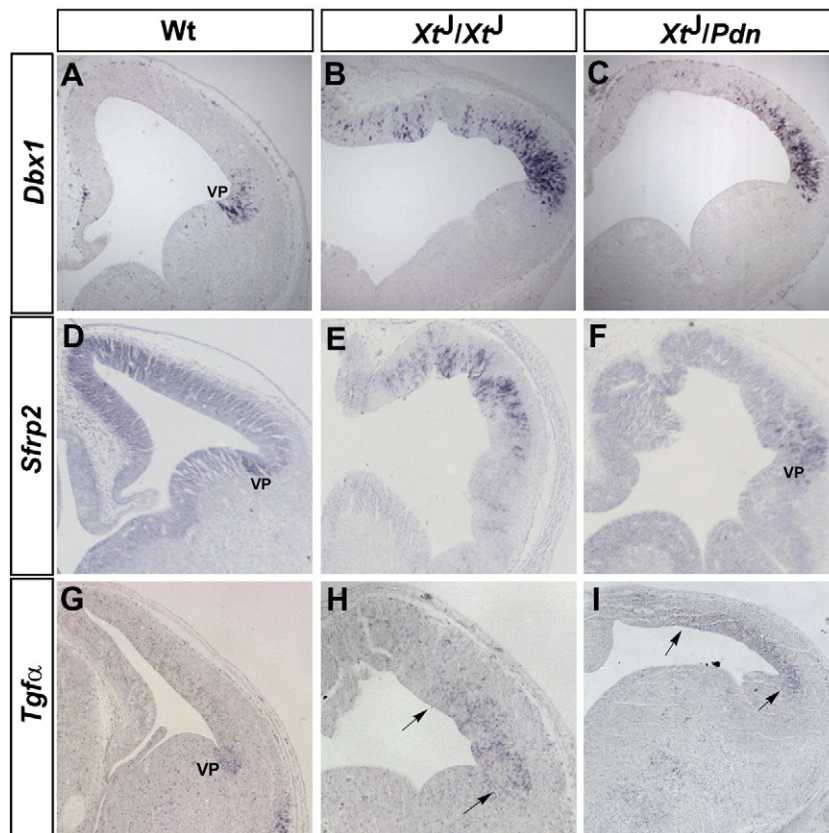
neocortex, however, regions where the laminar organization of *Tbr1*<sup>+</sup> layer VI neurons is severely disturbed correspond to areas with a dramatic disorganization of the glial scaffold (Figs. 8D–F). In contrast, in regions where the radial glia reach the pial surface *Tbr1*<sup>+</sup> layer VI neurons show a layered though more diffuse organization than in the wild-type cortex (Figs. 8G–I). This analysis therefore suggests that the disruption of the radial glial scaffold which is present already early in development causes at least some of the lamination defects in the *Gli3* mutant.

#### Expansion of *Dbx1* expression in *Gli3* mutants

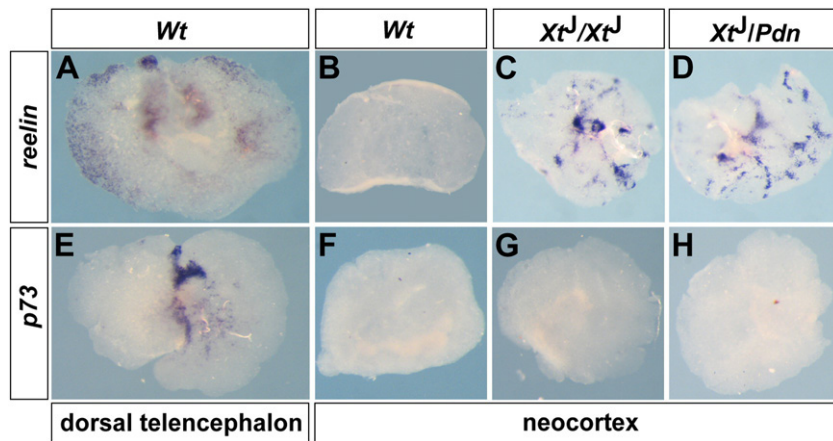
Despite the absence of the cortical hem *reelin* expressing cells are present in the *Gli3* mutant cortex (Fig. 3R and Theil, 2005). To address the potential origin of these cells, we analyzed *Dbx1* expression in *Gli3* mutants. Recently, *reelin*<sup>+</sup> cells have been reported to originate from *Dbx1*<sup>+</sup> progenitor cells in the septum and in the ventral pallidum (VP) (Bielle et al., 2005) raising the possibility that the *reelin*<sup>+</sup>/*p73*<sup>−</sup> cells in the *Gli3* mutant originate from such progenitors. As in *Gli3* mutants the rostral most dorsal telencephalon expresses ventral telencephalic marker genes (Kuschel et al., 2003; Tole et al., 2000b) we could not investigate whether *Dbx1* expression in the septum is affected by the *Gli3* mutation. However, we observed a widespread, though patchy expression of *Dbx1* in the VZ of both *Xt<sup>l</sup>/Xt<sup>l</sup>* and *Xt<sup>l</sup>/Pdn* embryos while *Dbx1* expression is confined to progenitor cells residing immediately at the PSB of E12.5 wild-type embryos (Figs. 9A–C). To investigate whether this ectopic *Dbx1* expression represents an expansion of the VP we performed in situ hybridization for *Sfrp2* and *Tgfa* which are co-expressed with *Dbx1* in the VP (Assimacopoulos et al., 2003; Kim et al., 2001). Interestingly, *Sfrp2* is ectopically expressed in groups of cells

within the *Xt<sup>l</sup>/Xt<sup>l</sup>* neocortex while its expression remains confined to the VP region of *Xt<sup>l</sup>/Pdn* embryos (Figs. 9D–F). In contrast, *Tgfa* expression expands into the neocortex of both mutants (Figs. 9G–I) suggesting that several VP markers are ectopically expressed in the *Gli3* mutant neocortex though to different extents.

The ectopic *Dbx1* expression might also suggest that an increased *Dbx1*<sup>+</sup> progenitor pool may give rise to the *reelin*<sup>+</sup> but *p73*<sup>−</sup> cells. To begin to address this hypothesis we employed an explant culture assay using wildtype E10.5 telencephalic tissue. This time point corresponds to the start of CR cell emigration from the cortical hem when only few CR cells have reached the neocortex (Muzio and Mallamaci, 2005). In a control experiment, we first tested whether the complete dorsal telencephalon of wildtype embryos can give rise to CR neurons under these conditions using in situ hybridization for *reelin* and *p73*. Indeed, we could detect two stripes of *reelin* expression in the centre of the explants and strong *reelin* expression at the lateral margins of the explants but only a few *reelin* expressing cells in the centres of the two telencephalic hemispheres which corresponds to neocortical tissue (*n*=4) (Fig. 10A). Also, *p73* expression is confined to the midline regions of the explant (*n*=4) (Fig. 10E). In the next set of experiments, we dissected just neocortical tissue excluding dorsal midline and VP tissue and analyzed the formation of *reelin* expressing cells after 48 h in vitro culture. In line with a previous report (Muzio and Mallamaci, 2005), neocortical tissue from wildtype embryos did not give rise to *reelin* or *p73* expressing cells (*n*=8 for both markers) (Figs. 10B, E). However, when we cultivated neocortical tissue from either *Xt<sup>l</sup>/Xt<sup>l</sup>* embryos or *Xt<sup>l</sup>/Pdn* embryos which, in contrast to the wildtype explant, expresses *Dbx1* we observed strong *reelin* but not *p73* expression in the explants (*n*=6 for both mutants and for both markers) (Figs. 10C, D, G, H). In combination with the ectopic *Dbx1*



**Fig. 9.** Ectopic *Dbx1* expression in the *Gli3* mutant neocortex. Coronal sections through the brains of E12.5 wild-type (A, D, G), *Xt<sup>l</sup>/Xt<sup>l</sup>* (B, E, H), and *Xt<sup>l</sup>/Pdn* (C, F, I) embryos. (A, D, G) *Dbx1*, *Sfrp2* and *Tgfa* expression in wild-type neocortex are confined to the VP area at the dorsal/ventral telencephalic boundary. (B, C) *Dbx1* is ectopically expressed in the VZ of the *Gli3* mutant neocortex. (E, F) *Sfrp2* is ectopically expressed in the *Xt<sup>l</sup>/Xt<sup>l</sup>* neocortex but not in the *Xt<sup>l</sup>/Pdn* mutant. (H, I) Expanded *Tgfa* expression domain in the *Gli3* mutant neocortex as indicated by arrows.



**Fig. 10.** *Gli3* mutant neocortex gives rise to *reelin*<sup>+</sup>/*p73*<sup>−</sup> cells in explant cultures. Explant cultures of E10.5 whole dorsal telencephalon (A, E) or neocortex (B–D, F–H) were hybridized with the indicated probes after 48 h of vitro culture. (A, E) Dorsal telencephalic explants give rise to *reelin* expressing cells in the centre of the explant and at its rostral and lateral margins. Note the region between the central *reelin* stripes and the margins has fewer *reelin*<sup>+</sup> cells. (A) *p73* expressing cells are only formed in the centre of the explant (E). (B–D) Wildtype neocortical explants do not form *reelin* expressing cells (B) while *Gli3* mutant explants show widespread *reelin* expression (C, D). (F–H) Neocortical explants from all genotypes lack *p73* expression.

expression, this result suggests that the ectopic *Dbx1*<sup>+</sup> progenitors may give rise to *reelin*<sup>+</sup> neurons in the *Gli3* mutants.

## Discussion

### Regionalization defects in the *Gli3* compound heterozygous mutant *Xt*<sup>l</sup>/*Pdn*

*Xt*<sup>l</sup>/*Xt*<sup>l</sup> embryos were previously shown to have severe defects in the regionalization of the telencephalon which are also present in *Xt*<sup>l</sup>/*Pdn* embryos but in a milder form. In the latter mutant, the expression of ventral telencephalic markers in dorsal locations occurs in a smaller domain and is restricted to the rostral most telencephalon (Kuschel et al., 2003). Dorsomedial structures are highly defective showing a morphological absence of the hippocampus and an overgrowth of choroid plexus tissue consistent with an altered balance between *Bmp* and *Wnt* signalling. In contrast to *Xt*<sup>l</sup>/*Xt*<sup>l</sup> embryos (Fotaki et al., 2006) the telencephalon and diencephalon are not fused and we could not find evidence for mixing of cells from both tissues. In addition to these findings, our analysis revealed a novel regionalization defect in *Gli3* mutants, namely an expansion of the VP into the lateral and dorsal pallium. This expansion may result from a lack of *Emx1* expression (Kuschel et al., 2003; Theil et al., 1999) as has been suggested previously (Medina et al., 2004; Puelles et al., 2000) or from a reduced *Lhx2* expression (Mangale et al., 2008). Alternatively, *Gli3* could play a general role in controlling *Dbx1* expression as ectopic *Dbx1* transcription was also found in the *Xt*<sup>l</sup>/*Xt*<sup>l</sup> spinal cord (Persson et al., 2002). Irrespective of the exact mechanism, these data indicate that the *Xt*<sup>l</sup>/*Pdn* telencephalon has similar but milder regionalization defects than *Xt*<sup>l</sup>/*Xt*<sup>l</sup> embryos consistent with *Pdn* being a hypomorphic *Gli3* allele. Our analysis also shows that except for this VP expansion the *Xt*<sup>l</sup>/*Pdn* neocortex is largely unaffected allowing us to investigate *Gli3* functions in layering.

### *Xt*<sup>l</sup>/*Pdn* mice show cortical lamination defects

Our analysis of cortical layering indicates a lamination phenotype in the *Xt*<sup>l</sup>/*Pdn* neocortex. The expression of several layer specific markers including the layer IV/V expression of *reelin* indicate a stronger dispersal of cortical neurons throughout the neocortex. This analysis also revealed areas with strong layering defects where cortical neurons were even positioned close to the ventricular surface. Given the mildly affected cortical lamination in hem ablated animals the finding of layering defects in the *Xt*<sup>l</sup>/*Pdn* neocortex comes as a

surprise especially as significant numbers of *reelin*<sup>+</sup> cells are present in the *Gli3* mutant MZ. Their molecular profile (*reelin*<sup>+</sup> *p73*<sup>−</sup> *Calretinin*<sup>−</sup>) and the fact that, unlike wildtype neocortical tissue, explants from mutant neocortex gives rise to *reelin*<sup>+</sup> but not *p73*<sup>+</sup> cells in an in vitro culture assay strongly suggests that these cells derive from the expanded *Dbx1*<sup>+</sup> progenitor pool in the mutant. The generation of these *reelin*<sup>+</sup> cells in the *Xt*<sup>l</sup>/*Pdn* neocortex but not in the hem ablated animals is likely to reflect differences in the timing of hem loss and concomitant changes in *Wnt* mediated patterning of the dorsal telencephalon and/or differences in patterning the VP (see above). Irrespective of the mechanism, these additional *reelin*<sup>+</sup> cells are not sufficient to drive radial migration of cortical neurons. Therefore, additional signalling pathways and their interaction with *reelin* signalling may underlie cortical lamination (Meyer et al., 2004; Yoshida et al., 2006). Furthermore, the *reelin*<sup>+</sup>/*p73*<sup>−</sup>/*Calretinin*<sup>−</sup> cell population might functionally differ from hem derived *reelin*<sup>+</sup> CR cells and might not be able to fully compensate for the loss of the latter cells (Bielle et al., 2005; Meyer et al., 2004). Collaboratively, these findings point at intrinsic functional differences between CR cell subpopulations. Such differences may be important for the establishment of different lamination patterns in distinct cortical regions.

### *Gli3* functions in cortical lamination

Except for a difference in timing, the *Gli3* mutation and hem ablation both lead to a loss of the cortical hem but have strikingly different effects on layering suggesting hitherto unknown roles for *Gli3* in lamination. The most striking observation of this manuscript relates to the rearrangement of *reelin*<sup>+</sup> and *Calretinin*<sup>+</sup> cells which initially show an even distribution over the cortical surface but cluster later in *Xt*<sup>l</sup>/*Pdn* embryos. These clusters could mechanically block access to the upper CP. Also, migrating neurons could be differentially exposed to *reelin* signals consistent with a recent report linking regular spaced clusters of CR cells in the immature presubicular cortex with the formation of vertical arrays of CP neurons (Nishikawa et al., 2002). More importantly, however, the radial glial network which serves as a guidance structure for migrating cortical neurons (Rakic, 2003) is severely disturbed in the vicinity of the *reelin*<sup>+</sup> cell clusters. A severe shortening of these processes and their detachment from the pial surface may lead to a failure to guide migrating neurons to the upper CP. Indeed, regions in the P0 *Xt*<sup>l</sup>/*Pdn* neocortex with the most severe lamination defects correlate with sites where the radial glial scaffold is severely disturbed. At present it is unknown whether the clustering of neurons in the MZ precedes the disorganization of the



radial glial scaffold or vice versa. However, as the disturbance of the radial glial network is only found locally while *Gli3* is expressed throughout the VZ it seems more likely that the formation of MZ clusters is the primary cause of the lamination phenotype.

The formation of these clusters could involve a role for *Gli3* in controlling the adhesive properties of neurons which are an important determinant in establishing cortical layers and are known to affect the spreading and distribution of reelin<sup>+</sup> and other MZ cell types (Borrell and Marin, 2006; Paredes et al., 2006). The formation of these clusters could also involve changes in adhesion as CR cells express specific cell adhesion molecules (Seki and Arai, 1991; Tsuru et al., 1996). Similarly, loss of Gli function in the spinal cord results in neuronal dispersal in the developing spinal cord (Bai et al., 2004; Lei et al., 2004; Wijgerde et al., 2002). As the molecules which control the adhesion of CP neurons and/or CR cells are currently unknown future work will have to address the identity of such factors.

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